

⑫ **EUROPEAN PATENT APPLICATION**
published in accordance with Art.
158(3) EPC

① Application number: 89909235.7

⑤ Int. Cl.⁵: C12N 9/02, C12N 15/00

② Date of filing: 09.08.89

⑧ International application number:
PCT/JP89/00811

⑦ International publication number:
WO 90/01542 (22.02.90 90/05)

④ Priority: 09.08.88 JP 199295/88
17.08.88 JP 204173/88

⑥ Date of publication of application:
19.09.90 Bulletin 90/38

③ Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

⑩ Applicant: **TORAY INDUSTRIES, INC.**
2-1, Nihonbashi Muromachi 2-chome
Chuo-ku
Tokyo 103(JP)

⑨ Inventor: **KAZAMI, Jun**
1-20, Tsunishi 2-chome
Kamakura-shi Kanagawa 248(JP)
Inventor: **NAKAMURA, Haruji**
11-27, Miyamatsu-cho
Hiratsuka-shi Kanagawa 254(JP)
Inventor: **GOTO, Toshio**
3-9, Yaguma 1-chome
Nakagawa-ku Nagoya-shi Aichi 454(JP)

⑫ Representative: **Kador & Partner**
Comellusstrasse 15
D-8000 München 5(DE)

⑬ **LUCIFERASE, LUCIFERASE-CODING GENE, AND PROCESS FOR PREPARING LUCIFERASE.**

⑭ Luciferase having the amino acid sequence of Fig. 1 and a gene coding it are disclosed. In addition, a recombinant vector DNA wherein the luciferase-coding gene is connected to the downstream portion of a promoter capable of expressing in each host cell, a transformant obtained by transforming each host cell by the vector DNA, and a process for preparing luciferase using such transformants are also disclosed.

EP 0 387 355 A1

S P E C I F I C A T I O N

Luciferase, Gene Encoding the Same and Production Process
of the Same

TECHNICAL FIELD

5 This invention relates to a purified enzyme
luciferase and a gene coding for the enzyme. This
invention further provides a novel recombinant vector DNA
in which the gene is inserted, a transformant containing
the vector DNA, and a process of producing luciferase
10 using the transformant.

BACKGROUND ART

Cypridina hilgendorffii is a marine ostracod
crusacean living in the coast of the Sea of Japan, which
releases a pale blue luminescent fluid when it is
15 disturbed. The luminescence is produced by the oxidation
of luciferin by an enzyme luciferase. The luminescent
system is very simple because another indispensable
component is not required unlike the luminescence of
firefly or luminescent bacteria, so that the application
20 of this luminescent system to the assay of a component
contained in a sample in a trace amount is expected.

 However, although luciferin can be chemically
synthesized in a large amount, luciferase cannot be
chemically synthesized because it is an enzyme, so that it
25 is difficult to obtain luciferase in a large amount.
This situation is also true in the luciferase of
Cypridina hilgendorffii and the highly purified luciferase

of *Cypridina hilgendorffii* has not yet been obtained.

Further, because of the sea pollution, the catch of *Cypridina hilgendorffii* drastically decreased. Thus, the constant supply of the luciferase of *Cypridina*

5 *hilgendorffii* is not assured. Therefore, it is desired to establish a large scale production process of the enzyme, which employs the genetic recombination technique.

The object of the present invention is to attain the synthesis of highly purified luciferase by chemical
10 synthesis process, or by genetic recombination process, to provide a gene encoding the protein, to attain the expression of the cloned gene in an animal cell, yeast cell, in *E. coli* cell or the like, and to produce the highly purified enzyme in a large amount using the cell.

15 DISCLOSURE OF THE INVENTION

The present invention provides luciferase with an amino acid sequence shown in Fig. 1, a gene encoding the amino acid sequence, a novel recombinant vector containing the gene, a transformant prepared by
20 transforming a host cell with the recombinant vector, and a process of producing luciferase using the transformant.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1a, 1b, 1c and 1d show the nucleotide sequence of the luciferase from *Cypridina hilgendorffii* as well as
25 the amino acid sequence thereof. The upper row in each line indicates the amino acid sequence and the lower row in each line indicates the nucleotide sequence of the

CDNA.

Fig. 2 shows a construction of a recombinant plasmid pCLO7 containing the cDNA encoding the luciferase from *Cypridina hilgendorffii* as well as the restriction map thereof.

Fig. 3 shows a construction of an expression vector pSVLCL5 of the luciferase from *Cypridina hilgendorffii* for animal cells.

Fig. 4a shows restriction maps of expression vectors pMEF3A, pMEF3B, pMEF3C and pMEF3D of the luciferase from *Cypridina hilgendorffii* for yeast cells and Fig. 4b shows the nucleotide sequence of the region in the vicinity of the junction region of a pheromone gene and cDNA of the luciferase, as well as the amino acid sequence thereof.

Fig. 5 shows a construction of an expression vector pGL1 of the luciferase from *Cypridina hilgendorffii* for yeast cells.

Fig. 6 shows a construction process of expression vectors pMT-CLP, pMT-CLS and pMT-CLT of the luciferase from *Cypridina hilgendorffii* for *E. coli*.

BEST MODE FOR CARRYING OUT THE INVENTION

The luciferase of the present invention is a protein containing 555 amino acids having an amino acid sequence of 1st to 555th amino acid in the amino acid sequence shown in Fig. 1, a protein containing 527 amino acids having an amino acid sequence starting from the 29th amino acid proline in Fig. 1, a protein containing 526

amino acids having an amino acid sequence starting from the 30th amino acid serine in Fig. 1, a protein containing 525 amino acids having an amino acid sequence starting from the 31st amino acid serine, or a protein
5 containing 524 amino acids having an amino acid sequence starting from the 32nd amino acid threonine. Further, the proteins having the same amino acid sequence of the above-mentioned proteins except for some substitution, deletion and/or insertion are included in the scope of
10 the present invention as long as they retain substantially the same luciferase activity. That is, luciferase equivalents are included in the scope of the present invention.

The gene of the present invention is a gene encoding
15 the above-described luciferase and has a DNA sequence shown in the lower row in Fig. 1. The DNAs having some substitution, deletion and/or insertion of the DNA sequence shown in Fig. 1 are also included within the scope of the present invention as long as substantially
20 the same luciferase activity is retained.

The procedure of obtaining the gene encoding the luciferase of the present invention will now be described. First, *Cypridina hilgendorffii* are disrupted in guanidine thiocyanate solution and total RNAs are
25 extracted therefrom, followed by purification of poly(A)⁺ RNAs by oligo(dT) cellulose column chromatography. After synthesizing cDNAs using the poly(A)⁺ RNAs, the cDNAs are

cloned into λ gt10 to obtain a cDNA library.

On the other hand, the amino acid sequence of the region in the vicinity of N-terminal of the luciferase protein purified from *Cypridina hilgendorffii* and the
5 amino acid sequences of the oligopeptides obtained by the digestion with lysylendopeptidase are determined and several oligonucleotides having nucleotide sequences corresponding to the determined sequences are chemically synthesized. These oligonucleotides are used as probes
10 for the screening of the above-described cDNA library.

The nucleotide sequence of the inserted gene in the recombinants which form a hybrid with the probes in the plaque hybridization is determined. If it matches with the amino acid sequence of the luciferase protein, the
15 inserted gene can be identified as a portion of the gene encoding the luciferase protein.

The present invention also provides recombinant vector DNAs containing each of the above-described DNAs ligated at a site downstream of a promoter by which the
20 gene can be expressed in a host cell such as animal cells, yeast cells and *E. coli* cells, the transformants transformed with the recombinant vector DNAs and processes of producing luciferase using the transformants.

25 More particularly, the recombinant vector DNAs of the present invention may be obtained by ligating the cDNA encoding the luciferase from *Cypridina hilgendorffii*

with a vector DNA which is stably maintained in animal cells, yeast cells or *E. coli* cells, which vector DNA contains a promoter by which the inserted gene can be expressed in the host cells.

5 The promoter is a signal for initiating the RNA synthesis, which is recognized by RNA polymerase and bound thereby. The DNA sequence downstream from the promoter is transcribed to mRNA. Thus, in order that the gene encoding the luciferase from *Cypridina hilgendorffii*
10 is transcribed to mRNA, it is necessary that the gene be located downstream of the promoter which functions in a host cell.

Thus, the recombinant vectors prepared by cleaving a vector DNA at an appropriate site downstream of the
15 promoter contained in the vector and inserting therein the DNA containing the gene encoding the luciferase may be utilized.

The promoter which is used herein may be any promoter as long as it functions in a host cell. For
20 example, promoters of animal genes and animal virus genes may be used for construction of the recombinant vector which functions in an animal cell. More particularly, examples of the promoters include SV40 late promoter, promoter of thymidine kinase gene, SV40 early promoter,
25 promoter of Cytomegalovirus and the like. For yeast cells, promoters of yeast genes may be employed. For example, promoters of repressible acid phosphatase gene

(*PHO5*), galactokinase gene (*GAL1*), a pheromone gene (*MFa1*) gene of yeast and the like may be employed. For *E. coli*, promoters of *E. coli* genes and *E. coli* phages genes may be employed. For example, the promoter of
5 lactose operon (*Lac*), the try operon promoter, the P_L promoter of λ phage and the like may be employed. Further, synthetic *tac* promoter and the like may also be employed.

Any vector DNA which is stably maintained in a host
10 cell and which has a promoter which functions in the host cell may be employed. For example, for animal cells, plasmid vectors and virus vectors may be employed. More particularly, pSV2 (a vector containing SV40 early promoter, *J. Mol. Appl. Genet. USA*, 1, 327 (1982)), pSVL
15 (a vector containing SV40 late promoter, commercially available from Pharmacia) and the like may be employed. For yeast cells, pMFa8 (a vector containing the promoter of a pheromone gene (*MFa1*), *Gene*, 3, 155 (1985)), pAM85 (a vector containing the promoter of repressible acid
20 phosphatase gene (*PHO5*), *Proc. Natl. Acad. Sci. USA*, 80, 1 (1983)) and the like may be employed. For *E. coli*, pMT-1 (originated from an expression vector pKM6 containing the promoter of *trp* operon (Japanese Laid Open Patent Application (Kokai) No. 61-247387), pUC18/pUC19
25 (*Gene*, 33, 103 (1985)) and the like may be employed.

By inserting the cDNA encoding luciferase downstream of a nucleotide sequence encoding a signal peptide for

protein secretion, which functions in the host cell, luciferase can be secreted to the outside of the cell. The signal sequence is not restricted to a specific one and the signal sequence of interleukin-2 (IL-2), for
5 example, may be employed for animal cells. For yeasts, the signal sequence of a pheromone and the like may be employed. For *E. coli*, the signal sequence of β -lactamase and the like may be employed. In cases where the luciferase is to be accumulated in the cells, it is
10 not necessary to ligate the signal sequence.

In cases where *E. coli* is used as the host cell and the produced luciferase is to be accumulated in the cell, it is necessary to attach a nucleotide sequence of "ATG" encoding methionine to the 5'-end of the gene which is
15 desired to be expressed, and to ligate the resulting gene having "ATG" at 5'-end at a site downstream of a promoter and an SD sequence, which function in *E. coli* cell. The SD sequence is a signal for the initiation of the protein synthesis from the "ATG" codon downstream thereof, which
20 sequence in mRNA is recognized and bound by ribosome. The reason why the methionine is attached is that most of eukaryotic genes encoding a protein to be secreted encodes the mature protein downstream of the signal sequence for the secretion of the protein so as to
25 produce a precursor protein having a signal peptide, and the mature protein is produced by cleaving off the signal peptide in the process of protein secretion, so that most

of the eukaryotic mature proteins do not contain methionine of which codon is indispensable to the initiation of the protein synthesis. Further, since the natural luciferase purified from *Cypridina hilgendorffii* is a mixture of two proteins of which N-terminals are serine and threonine, respectively, and since most of the eukaryotic signal sequence is cleaved next to alanine-X-alanine and a sequence of alanine-glutamic acid-alanine-proline exists in the amino acid sequence deduced from the nucleotide sequence of *Cypridina hilgendorffii* luciferase, three kinds of expression vector having a N-terminal region at the downstream of the methionine codon, which encodes the luciferase which starts from proline, serine and methionine, respectively are employed.

The transformants obtained by transforming a host cell such as animal cells, yeast cells and *E. coli* cells with each of the above-mentioned recombinant vectors are prepared by introducing the recombinant vector DNA into the host cell.

The animal cells which may be used in the present invention are not restricted. Examples of the animal cells include COS-1 cell (a cell transformed with SV40 from the kidney of Africa green monkey), CHO cell (originated from the ovary of Chinese Hamster) and the like, and COS-1 cell is preferred. The yeast cells which may be used in the present invention are not restricted.

Examples of the yeasts include *Saccharomyces cerevisiae*, *Shizosaccharomyces pombe*, *Pichia pastoris* and the like.

The *E. coli* cells which may be used in the present invention are not restricted and examples thereof include
5 HB101, JM109 and the like.

The method of introducing the recombinant vector DNA into the host cell is not restricted. For example, in cases where the host cell is an animal cell, DEAE-dextran method [Mol. Cell. Biol., 5, 1188 (1985)], calcium-
10 phosphate co-sedimentation method [Cell, 14, 725 (1978)], electroporation method [EMBO J. 1, 841 (1982)] or the like may be employed. Among these, DEAE-dextran method is preferred. In cases where the host cell is a yeast cell, protoplast method [Proc. Natl. Acad. Sci. USA, 75,
15 1929 (1978)] may preferably be employed. Further, in cases where the host cell is *E. coli*, calcium chloride method [J. Mol. Biol., 53, 154 (1970)] may preferably be employed.

By introducing each of the recombinant vector DNA
20 into the host cells, novel recombinant vector DNA in which the DNA containing the gene encoding the luciferase from *Cypridina hilgendorffii* as well as the transformants having the ability to produce the luciferase may be obtained.

25 Each of the transformants is cultured in a culture medium and the luciferase may be obtained from the culture. Any culturing medium may be employed as long as

the host cell can grow therein. For example, for animal cells, Dulbecco's modified Eagle medium or the like may be employed. For yeasts, YEPD medium (20 g/l of tryptone, 10 g/l of yeast extract and 20 g/ml of glucose) or the like may be employed. For *E. coli*, L broth (10 g/l of tryptone, 5 g/l of yeast extract and 10 g/l of sodium chloride) or the like may be employed.

Any culturing temperature may be employed as long as the cell can grow, and 15 - 45°C may usually be preferred. For animal cells and *E. coli* cells, 25 - 40°C is preferred and 30 - 37°C is more preferred. For yeasts, 15 - 45°C is preferred, and more preferably 20 - 30°C. The culturing period is not restricted and is usually 1 - 10 days, preferably 3 - 7 days for animal cells and yeasts, and 1 - 3 days for *E. coli*.

In cases where the promoter requires an appropriate induction, for example, in cases where the promoter is the promoter of metallothionein gene for animal cells, the promoter of repressible acid phosphatase gene for yeasts or *trp* promoter for *E. coli* or the like, the expression of the promoter may be induced by the manner required for the respective promoter such as addition of an appropriate inducer, removal of an appropriate substance, changing the culturing temperature, irradiation with ultraviolet light and the like. More particularly, in cases where *trp* promoter is employed for *E. coli*, the promoter can be induced by adding IAA

(indoleacrylic acid) which is an inducer of *trp* operon.

In cases where a trace amount of protein produced in the non-induced state adversely affects the growing of the cells, it is preferred that the expression of the promoter be repressed to a level as small as possible in the non-induced state. For example, a promoter of which expression is completely repressed in the non-induced state may be employed, or a repressor gene of the promoter may be co-employed. For example, in case of *trp* promoter, a recombinant plasmid having an repressor gene of the *trp* operon may preferably be employed. In this case, the tryptophane repressor gene (*trpR*) [Nucleic Acids Res. 8, 1552 (1980)] may be employed.

Alternatively, the above-described method for secreting the produced protein outside the cells may be employed.

The culture is separated into the supernatant and the cells by an appropriate method such as centrifugation, and the luciferase activity in the culture supernatant or in the cell extract is measured using a luminometer or the like. Although the culture supernatant or the cell extract may be used as it is as a crude enzyme solution, if required, the luciferase may be purified by, for example, the method by F. I. Tsuji [Methods in Enzymol., 57, 364 (1978)].

Examples

The present invention will now be described in more detail by way of examples thereof.

Example 1

Construction of cDNA Library

Five grams of *Cypridina hilgendorffii* collected at Tateyama Bay in Chiba prefecture which was stored in frozen state was suspended in 75 ml of a solution containing 6M guanidine thiocyanate, 5 mM sodium citrate (pH 7.0) and 0.5% sodium lauryl sarkosinate, and the suspension was homogenized with Polytron Homogenizer (commercially available from Chimanetica) to disrupt the cells. Lithium chloride solution (included in a kit commercially available from Amersham) was added thereto and about 600 µg of RNA was obtained by lithium chloride co-sedimentation method. Three hundred micrograms of aliquote of the thus obtained RNA was purified by oligo(dT) cellulose column (commercially available from Colaborative Research) chromatography to obtain about 15 µg of poly(A)⁺RNA. From 2 µg of the thus obtained poly(A)⁺RNA, 1 µg of double-stranded DNA was obtained using a cDNA synthesis kit (commercially available from Life Technologies, Inc). Internal EcoRI site of 0.15 µg of the thus obtained double-stranded DNA was protected by EcoRI methylase and an EcoRI linker was ligated using T4 DNA ligase. The resultant was digested with EcoRI to convert the both ends to EcoRI sites. The resulting DNA was inserted into the EcoRI site of λgt10 using T4 DNA ligase and the resultant was introduced into phage particles by the *in vitro* packaging method. *E. coli*

Example 2

5 After lyophilizing 100 µg of *Cypridina hilgendorffii*
luciferase which was purified by the method by F. I.
Tsuji [Methods in Enzymol., 57, 364 (1978)], the
resultant was dissolved in 100 µl of 0.1 M Tris-HCl (pH
7.6) containing 8M of urea and 0.14 M of 2-
10 mercaptoethanol and the solution was incubated at 37°C
for 3 hours to pyridylethylate the -SH groups. To the
resultant, were added 200 µl of 0.11 M Tris-HCl (pH 9.0),
1 µl of 2-methylmercaptoethanol and 1 µl of 2 µg/µl
lysylendopeptidase (commercially available from Wako Pure
15 Chemicals) and the resulting mixture was incubated at
37°C for 1 hour so as to allow the digestion. The
resultant was subjected to HPLC using VYDAC 218 TP54
(C₁₈) (commercially available from VYDAC) to separate
oligopeptides. Of the thus obtained oligopeptides, 13
20 oligopeptides were analyzed for the N-terminals by Amino
Acid Sequencer 470A (commercially available from Applied
Biosystem) to obtain the following 13 amino acid
sequences:

25 1 5 10

Thr-Cys-Gly-Ile-Cys-Gly-Asn-Tyr-Asn-Gln

Fragment 7-2

1 5 10

Glu-Gly-Glu-Cys-Ile-Asp-Thr-Arg-Cys-Ala-

11. 13

5 Thr-Cys-Lys

Fragment 12-1

1 5 10

Cys-Asn-Val-Cys-Tyr-Lys-Pro-Asp-Arg-Ile-

11

10 Ala

Fragment 12-2

1 5 7

Val-Ser-His-Arg-Asp-()-Glu

Fragment 13

15 1 5 10

Ala-Arg-Tyr-Gln-Phe-Gln-Gly-Pro-Met-Lys

(Cys)

Fragment 18

1 5 9

20 Arg-Phe-Asn-Phe-Gln-Glu-Pro-Gly-Lys

Fragment 21

1 5 10

Arg-Asp-Ile-Leu-Ser-Asp-Gly-Leu-Cys-Glu-

11 15

25 Asn-Lys-Pro-Gly-Lys

Fragment 23

1 5 10

Gly-Gln-Gln-Gly-Phe-Cys-Asp-His-Ala-Trp-

11 13

5 Glu-Phe-Lys

Fragment 27

1 5 10

Glu-Phe-Asp-Gly-Cys-Pro-Phe-Tyr-Gly-Asn-

11 15 18

10 Pro-Ser-Asp-Ile-Glu-Tyr-Cys-Lys

Fragment 38

1 5 10

Gly-Gly-Asp-()-Ser-Val-Thr-Leu-Thr-Met-

11 15 17

15 Glu-Asn-Leu-Asp-Gly-Gln-Lys

Fragment 40

1 5 10

His-Val-Leu-Phe-Asp-Tyr-Val-Glu-Thr-Cys-

11 15 20

20 Ala-Ala-Pro-Glu-Thr-Arg-Gly-Thr-Cys-Val-

21 25 30

Leu-Ser-Gly-His-Thr-Phe-Tyr-Asp-Thr-Phe

Fragment 47

1 5 10

25 Glu-Leu-Leu-Met-Ala-Ala-Asp-Cys-Tyr-()-

11 15 16

Asn-Thr-()-Asp-Val-Lys

1 5 10
()-Leu-Met-Glu-Pro-Tyr-Arg-Ala-Val-Cys-
11 15 20
()-Asn-Asn-Ile-Asn-Phe-Tyr-Tyr-Tyr-Thr

15	G	C	C	C	C	C
				A		A
				S		G

20

25

Probe II (corresponding to 6th - 10th amino acid sequence
of Fragment 23)

Cys-Asp-His-Ala-Trp

TGT GAT CAT GCT TGG

5 C C C C
 A
 G

10

3'-ACA CTA GTA CGI ACC-5'
G G G

Probe III (corresponding to 4th - 9th amino acid sequence
of Fragment 47)

Met-Ala-Ala-Asp-Cys-Tyr

15 ATG GCT GCT GAT TGT TAT

 C C C C C
 A A
 G G

20

3'-TAC CGI CGI CTA ACA AT-5'
G G

25

Probe IV (corresponding to third - 7th amino acid
sequence of Fragment 50)

Met-Glu-Pro-Tyr-Arg

ATG GAA CCT TAT CGT

5 G C C C
 A A
 G G
 AGA
 G

10

3'-TAC CTT GGI ATA TC-5'
 C G G

Probe V (corresponding to first - 10th amino acid
sequence of Fragment 13)

15 Ala-Arg-Tyr-Gln-Phe-Gln-Gly-Pro-Met-Lys

GCT CGT TAT CAA TTT CAA GGT CCT ATG AAA

 C C C G C G C C G
 A A A A
 G G G G
20 AGA
 G

3'-CGI GCI ATA GTT AAA GTT CCI GGI TAC TTT-5'
 T G C G C

25 One microgram each of the above-described 5
oligonucleotides was dissolved in 10 µl of 50 mM Tris-HCl
(pH 7.6) containing 10 mM magnesium chloride, 5 mM of

dithiothreitol, 1 mM of spermidine and 100 mM potassium chloride, and then 5 μ l of [γ - 32 P]ATP (3,000 Ci/mmol, commercially available from Amersham), 85 μ l of distilled water and 2 μ l of T4 polynucleotide kinase (commercially available from Takara Shuzo) were added thereto, followed by incubation at 37°C for 1 hour so as to carry out the labeling with 32 P.

Example 3

Screening of cDNA Library by Plaque Hybridization Method

About 10,000 plaques per one plate were formed on 50 agar plates using the cDNA library prepared in Example 1. The plaques were transferred to Nylon membranes and were denatured with 0.5 M sodium hydroxide/1.5 M sodium chloride solution, followed by neutralization in 0.5 M Tris-HCl (pH 7.0)/1.5 M sodium chloride. After incubating the membranes at 80°C for 2 hours to fix the phage DNAs to the membranes, prehybridization was performed by incubating the resulting membranes in 50 mM sodium phosphate (pH 7.4) containing 0.75 M sodium chloride, 5 x Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll and 0.1% polyvinylpyrrolidone), 5 mM EDTA, 0.1% SDS and 100 μ g/ml of denatured salmon sperm DNAs at 45°C for 2 hours.

Then the resulting membranes were transferred into a fresh solution with the same composition and oligonucleotide Probe V labelled in Example 2 was added thereto to a level of 5 μ Ci/ml, followed by incubation at

45°C overnight to carry out the hybridization. About 16 hours later, the membranes were washed with 6 x SSC [90 mM sodium citrate (pH 7.0)/0.9 M sodium chloride] containing 0.1% SDS twice for 30 minutes each at room temperature, and then twice for 30 minutes each at 45°C. After drying in the air, the membranes were autoradiographed at -70°C for 48 hours using X-CMAT AR(trademark, commercially available from Kodak).

The films were developed and 32 positive clones were obtained. Phage was grown from these positive clones on the agar plates and the phage DNAs were purified. The obtained DNAs were stored at -20°C.

Example 4

Comparison of Luciferase Protein and Primary Structure of the Gene Thereof

From the clone λ CL07 which contained the largest inserted fragment of about 1900 base pairs of the obtained 32 positive clones, the inserted fragment was cut out with restriction enzyme EcoRI and the fragment was subcloned into plasmid pUC18 to construct a recombinant plasmid pCL07 (Fig. 2). The nucleotide sequence of the 1.9 kb EcoRI fragment was determined by the usual dideoxy method. The determined nucleotide sequence is shown in Fig. 1.

By comparing the information of the obtained gene and of the protein obtained in Example 2, the protein matched with the primary structure of the gene as shown

in Table 1. As a result, the nucleotide sequence of the luciferase gene from *Cypridina hilgendorffii* as well as the amino acid sequence of the protein was determined as shown in Fig. 1.

5

10

15

20

25

Table 1
Correspondence between Amino Acid Sequence and Primary Structure of Gene

Results of Analysis of Amino Acid Sequence	Correspondence with Primary Structure of Gene
Fragment 7 - 1 Thr-Cys-Gly-Ile-Cys-Gly-Asn-Tyr-Asn-Gln	Thr-Cys-Gly-Ile-Cys-Gly-Asn-Tyr-Asn-Gln ACA TGC GGC ATA TGT GGT AAC TAT AAT CAA
Fragment 7 - 2 Glu-Gly-Glu-Cys-Ile-Asp-Thr-Arg-Cys-Ala- Thr-Cys-Lys	Glu-Gly-Glu-Cys-Ile-Asp-Thr-Arg-Cys-Ala- GAA GGA GAA TGT ATC GAT ACC AGA TGC GCA Thr-Cys-Lys ACA TGT AAA
Fragment 1 2 - 1 Cys-Asn-Val-Cys-Tyr-Lys-Pro-Asp-Arg-Ile- Ala	Cys-Asn-Val-Cys-Tyr-Lys-Pro-Asp-Arg-Ile- TGT AAT GTC TGC TAC AAG CCT GAC CGT ATT Ala GCA
Fragment 1 2 - 2 Val-Ser-His-Arg-Asp-()-Glu	Val-Ser-His-Arg-Asp-()-Glu GTT TCA CAT AGA GAT GTT GAG
Fragment 1 3 Ala-Arg-Tyr-Gln-Phe-Gln-Gly-Pro-Met-Lys (Cys)	Ala-Arg-Tyr-Gln-Phe-Gln-Gly-Pro-Met-Lys (Cys) GCC AGA TAT CAA TTC CAG GGC CCA TGC AAA
Fragment 1 8 Arg-Phe-Asn-Phe-Gln-Glu-Pro-Gly-Lys	Arg-Phe-Asn-Phe-Gln-Glu-Pro-Gly-Lys AGA TTT AAT TTT CAG GAA CCT GGT AAA
Fragment 2 1 Arg-Asp-Ile-Leu-Ser-Asp-Gly-Leu-Cys-Glu- Asn-Lys-Pro-Gly-Lys	Arg-Asp-Ile-Leu-Ser-Asp-Gly-Leu-Cys-Glu- CGA GAC ATA CTA TCA GAC GGA CTG TGT GAA Asn-Lys-Pro-Gly-Lys AAT AAA CCA GGG AAG
Fragment 2 3 Gly-Gln-Gln-Gly-Phe-Cys-Asp-His-Ala-Trp- Glu-Phe-Lys	Gly-Gln-Gln-Gly-Phe-Cys-Asp-His-Ala-Trp- GCA CAG CAA GGA TTC TGT GAC CAT GCT TGG Glu-Phe-Lys GAG TTC AAA

Table 1 (continued) Correspondence between Amino Acid Sequence and Primary Structure of Gene	
Results of Analysis of Amino Acid Sequence	Correspondence with Primary Structure of Gene
Fragment 2 7 Glu-Phe-Asp-Gly-Cys-Pro-Phe-Tyr-Gly-Asn- Pro-Ser-Asp-Ile-Glu-Tyr-Cys-Lys	Glu-Phe-Asp-Gly-Cys-Pro-Phe-Tyr-Gly-Asn GAG TTC GAC GGC TGC CCA TTC TAC GGG AAT Pro-Ser-Asp-Ile-Glu-Tyr-Cys-Lys CCT TCT GAT ATC GAA TAC TGC AAA
Fragment 3 8 Gly-Gly-Asp-()-Ser-Val-Thr-Leu-Thr-Met- Glu-Asn-Leu-Asp-Gly-Gln-Lys	Gly-Gly-Asp-()-Ser-Val-Thr-Leu-Thr-Met- GGT GGC GAC TGG TCT GTA ACC CTC ACC ATG Glu-Asn-Leu-Asp-Gly-Gln-Lys GAG AAT CTA GAT GGA CAG AAG
Fragment 4 0 His-Val-Leu-Phe-Asp-Tyr-Val-Glu-Thr-Cys- Ala-Ala-Pro-Glu-Thr-Arg-Gly-Thr-Cys-Val- Leu-Ser-Gly-His-Thr-Phe-Tyr-Asp-Thr-Phe-	His-Val-Leu-Phe-Asp-Tyr-Val-Glu-Thr-Cys- CAC GTC CTT TTC GAC TAT GTT GAG ACA TGC Ala-Ala-Pro-Glu-Thr-Arg-Gly-Thr-Cys-Val- GCT GCA CCG GAA ACG AGA GGA ACG TGT GTT Leu-Ser-Gly-His-Thr-Phe-Tyr-Asp-Thr-Phe- TTA TCA GGA CAT ACT TTC TAT GAC ACA TTC
Fragment 4 7 Glu-Leu-Leu-Met-Ala-Ala-Asp-Cys-Tyr-()- Asn-Thr-()-Asp-Val-Lys	Glu-Leu-Leu-Met-Ala-Ala-Asp-Cys-Tyr-()- GAG CTT CTG ATG GCC GCA GAC TGT TAC TGS Asn-Thr-()-Asp-Val-Lys AAC ACA TGG GAT GTA AAG
Fragment 5 0 ()-Leu-Met-Glu-Pro-Tyr-Arg-Ala-Val-Cys- ()-Asn-Asn-Ile-Asn-Phe-Tyr-Tyr-Tyr-Thr	()-Leu-Met-Glu-Pro-Tyr-Arg-Ala-Val-Cys- GGT CTC ATG GAG CCA TAC AGA GCT GTA TGT ()-Asn-Asn-Ile-Asn-Phe-Tyr-Tyr-Tyr-Thr CGT AAC AAT ATC AAC TTC TAC TAT TAC ACT

Example 5

Insertion of Luciferase cDNA into Expression Vector pSVL
Containing SV40 Late Promoter

One microgram of the above-mentioned 1.9 kb *EcoRI* fragment encoding luciferase from *Cypridina hilgendorffii* obtained in Example 4 was treated with 5 units of *E. coli* DNA polymerase I large fragment (commercially available from Takara Shuzo) in the presence of 1.5 mM each of dATP, dTTP, dCTP and dGTP to repair the ends of the fragment. On the other hand, vector pSVL (an expression vector containing SV40 late promoter, commercially available from Pharmacia) was digested with restriction enzyme *SmaI*.

Then the 1.9 kb fragment (0.3 µg) of which ends were repaired and the *SmaI* digest of pSVL (0.1 µg) were ligated by T4 DNA ligase, and *E. coli* HB101 competent cells (commercially available from Takara Shuzo) were transformed with the resulting reaction mixture to obtain a recombinant plasmid in which the 1.9 kb fragment was inserted. The obtained recombinant plasmid was named pSVLCL5 (Fig. 3).

Example 6

Production of Luciferase from *Cypridina hilgendorffii* by
COS-1 Cell

The expression vector pSVLCL5 (10 µg) constructed in Example 5 was introduced into COS-1 cells by DEAE-dextran method [Mol. Cell. Biol. 5, 1188 (1985)]. On the other

hand, as a control, pSVL (10 µg) was introduced in the same manner into COS-1 cells.

These cells were cultured in 10 ml of Dulbecco's modified Eagle Medium (commercially available from Nissui Pharmaceuticals) containing 10% fetal bovine serum in a culturing flask of 25 cm² in the presence of 5% CO₂ at 37°C for 5 days. During the culturing and after the culturing, 1 ml each of the culture liquid was recovered and was centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatant of each of them was collected to obtain culture supernatants.

After the culturing, cells were peeled from the flask by trypsin treatment and were washed with 1 ml of PBS (-) (commercially available from Nissui Pharmaceuticals). The washings were centrifuged at 3,000 rpm for 10 minutes at 4°C and the supernatant was discarded. This operation was further repeated twice and the cells were suspended in 200 µl of PBS(-). Freeze-thaw cycle was repeated three times to obtain a cell extract.

Example 7

Assay of Luciferase Activity Produced by Animal Cells

The luciferase activities in the culture supernatants described in Example 6 were measured by the following method and the results are shown in Table 2: That is, 30 µl of the culture supernatant and 270 µl of a measuring buffer [100 mM sodium phosphate (pH 7.0)/200 mM

sodium chloride] were mixed. To the mixture, was added 2 μ l of 33 μ M *Cypridina hilgendorffii* luciferin and the number of photons generated was counted immediately for 30 seconds using a luminometer (Lumac L2010). The
5 luminescent intensity is indicated in terms of the average number of photons per one second. The number of generated photons were measured in the same manner for the culture supernatant of COS-1 cell in which pSLV was introduced as a control.

10 The luciferase activity in the cell extract described in Example 6 was measured by the following method and the results are shown in Table 2: That is, 10 μ l of the cell fraction prepared in Example 6 and 290 μ l of the above-described measuring buffer were mixed and 2
15 μ l of 33 μ M *Cypridina hilgendorffii* luciferin was added thereto, followed by the measurement of luciferase activity in the same manner as in the measurement for the culture supernatants.

20

25

Table 2

Activity of Luciferase ($\times 10^6$ cps/ml)						
plasmid	Extracellular				Intracellular	
	24 hours	48 hours	72 hours	96 hours	120 hours	120 hours
(a) pSVLCL5 (No. 1)	2.2	4.0	4.3	4.5	5.2	1.2
(b) pSVLCL5 (No. 2)	2.3	5.8	8.3	9.0	10.5	3.0
(c) pSVLCL5 (No. 3)	2.1	3.1	3.8	4.1	5.5	6.8
(d) pSVLCL5 (No. 4)	2.3	4.0	5.5	5.7	6.7	1.4
(e) pSVL (control)	2.0	2.5	2.3	2.3	2.1	0.2

Example 8

Synthesis of Oligonucleotides for Yeast Expression Vector and Annealing

Luciferase proteins having the amino acid sequence starting from the 29th amino acid proline of the amino acid sequence shown in Fig. 1 (YP type), from the 30th amino acid serine (YN type), from the 31st amino acid serine (YS type) and from the 32nd amino acid threonine (YT type), respectively, were prepared since (1) the wild type luciferase purified from *Cypridina hilgendorffii* is a mixture of two proteins of which N-terminal is the 31st amino acid serine in the amino acid sequence shown in Fig. 1 and the 32nd amino acid threonine; (2) an amino acid sequence having the characteristics of the signal sequence for the secretion of proteins exists at the N-terminal of the amino acid sequence of the luciferase, which is deduced from the nucleotide sequence of the cDNA; and since (3) the signal sequence is cleaved off at the downstream of the sequence of alanine-X-alanine in most of eukaryotes and *Cypridina hilgendorffii* luciferase has a sequence of alanine-glutamic acid-alanine-proline. To ligate the proteins downstream of the signal sequence of the α pheromone, the following 10 oligonucleotides were synthesized.

25	YP-1	5'-CCTTCAAGTACTCCA-3'
	YP-2	5'-CTGTTGGAGTACTTCAAGG-3'
	YS-1	5'-AGTACACCA-3'

- YS-2 5'-CTGTTGGTGTACT-3'
- YT-1 5'-ACTCCA-3'
- YT-2 5'-CTGTTGGAGT-3'
- YN-1 5'-TCGTCGACACCA-3'
- 5 YN-2 5'-CTGTTGGTGTGACGA-3'
- U-1 5'-ACAGTCCCAACATCTTGTGAAGCTAAAGAAGGAGA
ATGTAT-3'
- U-2 5'-CGATACATTCTCCTTCTTTAGCTTCACAAGATG
TTGGGA-3'
- 10 5'-Ends of the synthetic oligonucleotides YP-2, YS-
2, YT-2, YN-2 and U-2 were phosphorylated by T4 DNA
kinase. That is, 300 pmol each of the oligonucleotides
was reacted in 20 µl of a reaction mixture [50 mM Tris-
HCl (pH 7.6) containing 10 mM magnesium chloride, 0.1 mM
15 spermidine, 5 mM dithiothreitol and 0.1 mM EDTA] in the
presence of 10 units of T4 DNA kinase (commercially
available from Takara Shuzo) at 37°C for 1 hour and then
the reaction mixture was heated at 70°C for 5 minutes,
followed by storage at -20°C.
- 20 The annealing of each oligonucleotide was performed
as follows:
- For YP type, 50 pmol each of YP-1, phosphorylated
YP-2, U-1 and phosphorylated U-2 were mixed. For YS
type, 50 pmol each of YS-1, phosphorylated YS-2, U-1 and
25 phosphorylated U-2 were mixed. For YT type, 50 pmol each
of YT-1, phosphorylated YT-2, U-1 and phosphorylated U-2
were mixed. For YN type, 50 pmol each of YN-1,

phosphorylated YN-2, U-1 and phosphorylated U-2 were mixed. Each mixture was heated at 70°C for 5 minutes and then the power of the incubator was shut off to leave the mixture to stand until the temperature is lowered to
5 42°C.

Example 9

Insertion of Luciferase cDNA into Expression Vector pMFa8 Containing the Promoter of Yeast α Pheromone Gene

The synthetic oligomers described in Example 8 were
10 respectively inserted into *Cypridina hilgendorffii* luciferase cDNA at the *Cla*I site to construct luciferase cDNAs having *Stu*I site at the 5'-end, from which 28, 29, 30 and 31 amino acids from the N-terminal were cut off, respectively.

15 The expression vector pMFa8 for yeasts [Gene, 3, 155 (1985): ATCC 37418] was digested with restriction enzyme *Stu*I immediately downstream of the region encoding the leader sequence of the α pheromone gene and the above-mentioned luciferase cDNA was inserted therein. The thus
20 constructed expression vectors were named pMEF3A (YP type), pMEF3B (YS type), pMEF3C (YT type) and pMEF3D (YN type), respectively (Fig. 4a).

The nucleotide sequence in the vicinity of the junction region between the α pheromone gene and
25 luciferase cDNA of each expression vector was checked by the usual dideoxy method using a sequence in the luciferase cDNA, 5'-TATAAATGGTCCAAGGA-3', as a primer to

confirm that the cDNAs were inserted correctly. The nucleotide sequences in the vicinity of the junction region between the α pheromone gene and luciferase cDNA of pMFE 3A, pMFE3B, pMFE3C and pMFE3D are shown in Fig.

5 4b.

Example 10

Insertion of Luciferase cDNA into Expression Vector p103
Containing the Promoter of Yeast GAL1 Gene

The two EcoRI fragments with a size of 1.3 kb and
10 0.6 kb were cut out from λ CL07 obtained in Example 3 and
were respectively subcloned to plasmid pUC18 to construct
plasmids pCL0712 and pCL0742, respectively. pCL07 (1 μ g)
and pCL0712 (1 μ g) were cut with HindIII and BglII, and a
DNA fragment containing the N-terminal of the luciferase
15 was purified from pCL07 and a DNA fragment containing the
C-terminal of the luciferase was purified from pCL0712.
The two fragments were subcloned to a plasmid pSPT18
(commercially available from Boehringer-Mannheim) at the
HindIII site thereof, and the obtained plasmid was named
20 pSTCL81.

The pSTCL81 (1 μ g) was digested with BamHI and the
total cloned cDNA sequence was obtained as BamHI
fragment.

On the other hand, about 1 μ g of expression vector
25 p103 [containing a polylinker including BamHI site at the
downstream of the GAL1 promoter of *Saccharomyces*
cerevisiae (Mol. Cell. Biol., 4, 1440 (1984)); presented

by Assistant Professor Shun Harajima of Osaka University] was digested with BamHI and the resultant was ligated with the about 0.1 µg of the above-mentioned cDNA fragment to construct an expression vector pGL1 in which the luciferase cDNA was inserted downstream of the GAL1 promoter (Fig. 5).

Example 11

Production of Luciferase from *Cypridina hilgendorffii* by Yeast

- 10 Ten micrograms each of the expression vectors pMFE3A, pMFE3B, pMFE3C and pMFE3D prepared in Example 9 were introduced into *Saccharomyces cerevisiae* 20B-12 strain [Gene, 37, 155 (1985)] by the protoplast method [Proc. Natl. Acad. Sci. USA, 75, 1929 (1978)].
- 15 These transformants were cultured at 30°C for 3 days in 100 ml of YEPD medium contained in a 1-liter culturing flask. During the culturing and after the culturing, 5 ml each of the culture was collected and was centrifuged at 4°C for 10 minutes at 3000 rpm. The supernatants were
- 20 collected to obtain culture supernatants.

- The cells harvested from one milliliter of the each culture was washed with 5 ml of sterilized distilled water, and the cells were suspended in 1 ml of 50 mM sodium phosphate (pH 7.5) containing 0.1% Triton X-100.
- 25 To this suspension, 1 ml of a glass beads (0.45 mm diameter) suspension was added and the mixture was left to stand at 0°C for 5 minutes while sometimes vigorously

agitating the mixture with a mixer. The glass beads were separated by gentle centrifugation, and the supernatant was transferred to a 1.5 ml Eppendorf's tube, followed by centrifugation at 15,000 rpm for 5 minutes. The obtained
5 supernatant was used as the cell extract.

Example 12

Production of Luciferase from *Cypridina hilgendorffii* by Yeast

The expression vector pGL1 (10 µg) was introduced
10 into *Saccharomyces cerevisiae* YSH2676 strain ((a) ura3-52 leu2-3 leu2-112 trp1 pho3 pho5 his1-29) by the protoplast method as in Example 11.

The transformant was cultured at 30°C for 2 days in 100 ml of a medium (1% yeast extract, 2% peptone and 2%
15 galactose) in a 1-liter culturing flask. During the culturing and after the culturing, 5 ml each of the culture was collected and was centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatants were recovered and were used as the culture supernatant.

20 Further, the cell extract was prepared in the same manner as in Example 11.

Example 13

Assay of Activity of Luciferase Produced by Yeast

The luciferase activities in the culture
25 supernatants described in Example 11 were measured in the same manner as in the measurement for the culture supernatants of the animal cells described in Example 7.

The results are shown in Table 3. As a control, the number of generated photons of the culture supernatant of *S. cerevisiae* 208-12 strain into which pMFa8 was introduced was also counted in the same manner.

5 The luciferase activities in the yeast cells described in Example 11 were performed by the method described below and the results are shown in Table 3. That is, 10 μ l of the cell extract prepared in Example 11 and 290 μ l of the above-described measuring buffer were
10 mixed and 2 μ l of 33 μ M *Cypridina hilgendorffii* luciferin was added thereto, followed by the measurement of the luciferase activity in the same manner as in the measurement for the culture supernatants.

15

20

25

Table 3

		Activity of Luciferase ($\times 10^5$ c p s / m l)				
plasmid		12 hours	21 hours	38 hours	47 hours	54 hours
(a) pMFE3A	Intracellular	<0.01	<0.01	0.01	0.02	0.01
	Extracellular	0.05	0.02	4.84	13.47	2.11
(b) pMFE3B	Intracellular	<0.01	<0.01	0.02	0.01	<0.01
	Extracellular	0.06	0.20	5.22	2.73	1.02
(c) pMFE3C	Intracellular	<0.01	<0.01	0.02	0.01	0.01
	Extracellular	0.10	0.21	2.76	0.79	0.89
(d) pMFE3D	Intracellular	<0.01	<0.01	0.02	0.01	0.01
	Extracellular	0.06	0.21	3.97	0.76	1.02
(e) control	Intracellular	<0.01	<0.01	<0.01	0.01	<0.01
	Extracellular	0.06	0.04	0.05	0.06	0.11

Example 14

Assay of Activity of Luciferase Produced by Yeast

The luciferase activities in the culture supernatants were determined in the same manner as in the measurement for the culture supernatant of the animal cells described in Example 7, and the results are shown in Table 4. As a control, the number of generated photons of the culture supernatant of *S. cerevisiae* YSH2676 strain into which p103 was introduced was also counted in the same manner.

The luciferase activities in the yeast cells described in Example 12 were measured in the same manner as in Example 13, and the results are shown in Table 4.

15

20

25

Table 4

		Activity of Luciferase ($\times 10^5$ cps/ml)		
clone No.		20 hours	43 hours	51 hours
(a) No. 1	Intracellular	0.06	0.07	0.07
	Extracellular	0.53	7.28	7.71
(b) No. 2	Intracellular	0.04	0.06	0.07
	Extracellular	0.44	3.04	3.49
(c) No. 3	Intracellular	0.07	0.07	0.06
	Extracellular	0.40	3.00	4.70
(d) No. 4	Intracellular	0.05	0.10	0.09
	Extracellular	0.92	5.89	6.27
(e) No. 5	Intracellular	0.05	0.08	0.05
	Extracellular	0.50	2.52	2.47
(f) control	Intracellular	0.01	n.t.	n.t.
	Extracellular	0.08	0.13	0.03

Example 15

Synthesis of Oligonucleotides for *E. coli* ExpressionVector and Annealing

To construct expression vectors containing a gene
5 encoding the luciferase of which amino acid sequence
starts from the sequence of methionine-proline (EP type),
methionine-serine (ES type) or methionine-threonine (ET
type) at a site downstream of the promoter and an SD
sequence of the *E. coli* tryptophan synthesis gene (*trp*)
10 operon, the following 6 oligonucleotides were
synthesized:

EP-1	5'-CGATGCCGTCAAGTACACCA-3'
EP-2	5'-CTGTTGGTGTACTTGACGGCAT-3'
ES-1	5'-CGATGAGTACACCA-3'
15 ES-2	5'-CTGTTGGTGTACTCAT-3'
ET-1	5'-CGATGACACCA-3'
ET-2	5'-CTGTTGGTGTCAT-3'

The N-terminals of 300 pmol each of the synthetic
oligonucleotides EP-2, ES-2 and ET-2 as well as U-2
20 prepared in Example 8 were phosphorylated using T4 DNA
kinase as in Example 8 and the phosphorylated
oligonucleotides were stored at -20°C.

For EP type, 50 pmol each of EP-1, phosphorylated
EP-2, U-1 and phosphorylated U-2 were mixed. For ES
25 type, 50 pmol each of ES-1, phosphorylated ES-2, U-1 and
phosphorylated U-2 were mixed. For ET type, 50 pmol each
of ET-1, phosphorylated ET-2, U-1 and phosphorylated U-2

were mixed. Each of the mixtures was subjected to annealing as in Example 8.

Example 16

Insertion of Luciferase cDNA into Expression Vector pMT1
5 containing *E. coli* trp Promoter

Expression vector pMT-1 [originated from pKM6 (Japanese Laid Open Patent Application (Kokai) No. 61-247387)] having the promoter and an SD sequence of *E. coli* tryptophan operon (*trp*) was digested with
10 restriction enzymes *Sma*I, *Cla*I and *Pvu*II.

On the other hand, the expression vector pCLO7 prepared in Example 3 was digested with *Sma*I and *Cla*I, and a DNA fragment containing luciferase cDNA downstream from the *Cla*I site was separated and purified by the
15 agarose gel electrophoresis method.

Using T4 DNA ligase (commercially available from Takara Shuzo), 0.1 µg each of the pMT-1 digest and the purified fragment from pCLO7 were ligated and the resultant was digested again by restriction enzyme *Sma*I.
20 *E. coli* HB101 competent cells (commercially available from Takara Shuzo) was transformed with the resultant to construct a plasmid pMT-CLO7. This plasmid had a part of the luciferase cDNA of the region downstream from the *Cla*I site, at a site downstream of the *trp* promoter/SD
25 sequence.

The plasmid pMT-CLO7 was digested with restriction enzyme *Cla*I and 0.1 µg of the obtained digest and 5 µl of

the synthetic DNA construct in Example 15 were ligated by T4 DNA ligase to construct expression vectors containing the luciferase gene starting from the codons of methionine-proline (EP type), methionine-serine (ES type) or methionine-threonine (ET type), at a site downstream of the *trp* promoter/SD sequence. The thus constructed plasmids were named pMT-CLP, pMT-CLS and pMT-CLT, respectively.

The nucleotide sequence in the vicinity of the junction region between the SD sequence and luciferase gene of each expression vector was checked by the usual dideoxy method using a sequence of 5'-TATAAATGGTCCAAGGA-3' in the luciferase cDNA as a primer to confirm that the cDNA was inserted correctly.

The restriction maps of pMT-CLP, pMT-CLS and pMT-CLT as well as the confirmed nucleotide sequences are shown in Fig. 6.

Example 17

Production of Luciferase from *Cypridina hilgendorffii* by *E. coli*

E. coli HB101 was transformed with each expression vector prepared in Example 16, and the obtained each transformant was cultured statically in 5 ml of L broth (containing 100 mg/l of ampicillin) overnight at 37°C. On the next day, 1 ml of the culture fluid was collected and was suspended in 50 ml of a synthetic medium [2 x M9-casamino acids medium (6 g/l of potassium dihydrogen

phosphate, 12 g/l of disodium hydrogen phosphate, 10 g/l of casamino acids, 10 g/l of sodium chloride, 1 g/l of ammonium chloride), 1 mg/l of thiamine-HCl, 250 mg/l of magnesium sulfate, 1% glucose and 100 mg/l of ampicillin, and the resultant was cultured overnight at 25°C with shaking. On the morning of the next day, IAA (final concentration of 20 mg/l) and glucose (final concentration of 1%) were added and the pH thereof was adjusted to 7.5 with 12.5% ammonia water. The culture was continued for 3 hours at 25°C. After 3 hours, IAA, glucose and ammonia water were added in the same manner and the culture was continued for another 3 hours. After the culturing, 8 ml of the culture fluid was centrifuged to collect the cells, and the cells were suspended in 0.5 ml TE buffer [10 mM Tris-HCl (pH 8.0)/1 mM EDTA]. Freeze-thaw cycle was repeated 3 times using warm water at 42°C and dry ice/acetone to disrupt the cells and the resultant was centrifuged at 10,000 rpm for 10 minutes. The obtained supernatant was used as a crude enzyme solution.

Example 18

Assay of Activity of Luciferase Produced by *E. coli*

The luciferase activity in the crude enzyme solution prepared in Example 17 was measured by the method described below and the results are shown in Table 5. That is, 150 μ l of the crude enzyme solution and 150 μ l of the measuring buffer and 2 μ l of 33 μ M Cypridina

hilgendorffii luciferin were mixed and the number of generated photons were counted for 30 seconds. The results are shown in Table 5. As a control, the number of the generated photons were counted for *E. coli* HB101 in which pMT-CLR (a plasmid in which the synthetic DNA is inserted in the wrong orientation).

Table 5

	Plasmid	Luciferase Activity (cps)
10	(a) pMT-CLP	1200
	(b) pMT-CLS	870
	(c) pMT-CLT	540
	(d) pMT-CLR	200
15	(control)	

INDUSTRIAL APPLICABILITY

The luciferase from *Cypridina hilgendorffii* provides a luminescent system with very high luminescence intensity. Therefore, the enzyme may be attached to an antibody molecule and used for EIA (enzyme immunoassay). Alternatively, the enzyme may be attached to DNA/RNA molecule which may be used in the DNA probe method. Thus, the wide use of the enzyme for various assays is expected.

By the present invention, the primary structure of the cDNA encoding the luciferase from *Cypridina*

hilgendorffii was determined and the primary structure of the luciferase was also identified. By culturing the animal cells, yeasts or *E. coli* containing the expression vector of the luciferase of the present invention in a
5 large scale, the luciferase may be supplied constantly in a large amount at a low cost.

Further, the methodology for the promotion of the stability of the luciferase, improvement of the quantum yield of the luminescence photons, improvement of the
10 luminescence conditions and for the change in the luminescence wavelength by employing protein engineering technique was developed.

15

20

25

CLAIMS

- (1) Purified luciferase having an amino acid sequence of 1st to 555th amino acid in the amino acid sequence shown in Fig. 1 and equivalents thereof.
- 5 (2) Purified luciferase having an amino acid sequence of 29th to 555th amino acid in the amino acid sequence shown in Fig. 1 and equivalents thereof.
- (3) Purified luciferase having an amino acid sequence of 30th to 555th amino acid in the amino acid sequence shown
10 in Fig. 1 and equivalents thereof.
- (4) Purified luciferase having an amino acid sequence of 31st to 555th amino acid in the amino acid sequence shown in Fig. 1 and equivalents thereof.
- (5) Purified luciferase having an amino acid sequence of
15 32nd to 555th amino acid in the amino acid sequence shown in Fig. 1 and equivalents thereof.
- (6) A gene encoding luciferase or an equivalent thereof according to any one of claims 1 - 5.
- (7) The gene of claim 6 having a nucleotide sequence shown in
20 Fig. 1.
- (8) A recombinant vector DNA comprising the gene of claim 6 ligated at a site downstream of a promoter which can be expressed in a host cell.
- (9) A recombinant vector DNA comprising the gene of
25 claim 6 ligated at a site downstream of a promoter and an SD sequence, which can be expressed in *E. coli*.
- (10) A transformant prepared by transforming a host cell

with the vector DNA of claim 8 or 9.

(11) The transformant of claim 10 which is an animal cell, a yeast cell or *E. coli* cell.

(12) A process of producing luciferase comprising
5 culturing the transformant of claim 10 or 11.

10

15

20

25

FIG. 1a

Met	Lys	Leu	Ile	Ile	Leu	Ser	Ile	Ile	Leu	Ala	Tyr	Cys	Val	Thr	Val	Asn	Cys	Gln	Asp	10	20
ATG	AAG	CTA	ATA	AAT	CTG	TCT	ATT	ATA	TTG	GCC	TAC	TGT	GTC	ACA	GTC	AAC	TGC	CAG	GAT	10	20
																				30	40
																				50	60
Ala	Cys	Pro	Val	Glu	Ala	Glu	Ala	Pro	Ser	Ser	Thr	Pro	Thr	Val	Pro	Thr	Ser	Cys	Glu	70	80
GCA	TGT	CCT	GTA	GAA	GCT	GAA	GCA	CCG	TCA	AGT	ACA	CCA	ACA	GTC	CCA	ACA	TCT	TGT	GAA	70	80
																				90	100
																				110	120
Ala	Lys	Glu	Gly	Glu	Cys	Ile	Asp	Thr	Arg	Cys	Ala	Thr	Cys	Lys	Arg	Asp	Ile	Leu	Ser	130	140
GCT	AAA	GAA	GGA	GAA	TGT	ATC	GAT	ACC	AGA	TGC	GCA	ACA	TGT	AAA	CGA	GAC	ATA	CTA	TCA	130	140
																				150	160
																				170	180
Asp	Gly	Leu	Cys	Glu	Asn	Lys	Pro	Gly	Lys	Thr	Cys	Cys	Arg	Met	Cys	Gln	Tyr	Val	Ile	190	200
GAC	GGA	CTG	TGT	GAA	AAT	AAA	CCA	GGG	AAG	ACA	TGC	TGT	AGA	ATG	TGC	CAG	TAT	GTA	ATT	190	200
																				210	220
																				230	240
Glu	Cys	Arg	Val	Glu	Ala	Ala	Gly	Tyr	Phe	Arg	Thr	Phe	Tyr	Gly	Lys	Arg	Phe	Asn	Phe	250	260
GAA	TGC	AGA	GTA	GAA	GCT	GCT	GGA	TAT	TTT	AGA	ACG	TTT	TAC	GGC	AAA	AGA	TTT	AAT	TTT	250	260
																				270	280
																				290	300
Gln	Glu	Pro	Gly	Lys	Tyr	Val	Leu	Ala	Arg	Gly	Thr	Lys	Gly	Gly	Asp	Trp	Ser	Val	Thr	310	320
CAG	GAA	CCT	GGT	AAA	TAT	GTG	CTG	GCT	CGA	GGA	ACC	AAG	GGT	GGC	GAC	TGG	TCT	GTA	ACC	310	320
																				330	340
																				350	360
Leu	Thr	Met	Glu	Asn	Leu	Asp	Gly	Gln	Lys	Gly	Ala	Val	Leu	Thr	Lys	Thr	Thr	Leu	Glu	370	380
CTC	ACC	ATG	GAG	AAT	CTA	GAT	GGA	CAG	AAG	GGA	GCT	GTA	CTG	ACT	AAG	ACA	ACA	CTG	GAG	370	380
																				390	400
																				410	420
Val	Val	Gly	Asp	Val	Ile	Asp	Ile	Thr	Gln	Ala	Thr	Ala	Asp	Pro	Ile	Thr	Val	Asn	Gly	430	440
GTA	GTA	GGA	GAC	GTA	ATA	GAC	ATT	ACT	CAA	GCT	ACT	GCA	GAT	CCT	ATC	ACA	GTT	AAC	GGA	430	440
																				450	460
																				470	480

FIG. 1b

															170		180		
Gly	Ala	Asp	Pro	Val	Ile	Ala	Asn	Pro	Phe	Thr	Ile	Gly	Glu	Val	Thr	Ile	Ala	Val	Val
GGA	GCT	GAC	CCA	GTT	ATC	GCT	AAC	CCG	TTC	ACA	ATT	GGT	GAG	GTG	ACC	ATT	GCT	GTT	GTG
	490				500				510				520			530			540
															190		200		
Glu	Ile	Pro	Gly	Phe	Asn	Ile	Thr	Val	Ile	Glu	Phe	Phe	Lys	Leu	Ile	Val	Ile	Asp	Ile
GAA	ATA	CCC	GGC	TTC	AAT	ATT	ACA	GTC	ATC	GAA	TTC	TTT	AAA	CTA	ATC	GTG	ATT	GAT	ATT
	550				560				570				580			590			600
															210		220		
Leu	Gly	Gly	Arg	Ser	Val	Arg	Ile	Ala	Pro	Asp	Thr	Ala	Asn	Lys	Gly	Leu	Ile	Ser	Gly
CTG	GGA	GGA	AGA	TCT	GTG	AGA	ATT	GCT	CCA	GAC	ACA	GCA	AAC	AAA	GGA	CTG	ATA	TCT	GGT
	610				620				630				640			650			660
															230		240		
Ile	Cys	Gly	Asn	Leu	Glu	Met	Asn	Asp	Ala	Asp	Asp	Phe	Thr	Thr	Asp	Ala	Asp	Gln	Leu
ATC	TGT	GGT	AAT	CTG	GAG	ATG	AAT	GAC	GCT	GAT	GAC	TTT	ACT	ACA	GAC	GCA	GAT	CAG	CTG
	670				680				690				700			710			720
															250		260		
Ala	Ile	Gln	Pro	Asn	Ile	Asn	Lys	Glu	Phe	Asp	Gly	Cys	Pro	Phe	Tyr	Gly	Asn	Pro	Ser
GCG	ATC	CAA	CCC	AAC	ATA	AAC	AAA	GAG	TTC	GAC	GGC	TGC	CCA	TTC	TAC	GGG	AAT	TCT	TCT
	730				740				750				760			770			780
															270		280		
Asp	Ile	Glu	Tyr	Cys	Lys	Gly	Leu	Met	Glu	Pro	Tyr	Arg	Ala	Val	Cys	Arg	Asn	Asn	Ile
GAT	ATC	GAA	TAC	TGC	AAA	GGT	CTC	ATG	GAG	CCA	TAC	AGA	GCT	GTA	TGT	CGT	AAC	AAT	ATC
	790				800				810				820			830			840
															290		300		
Asn	Phe	Tyr	Tyr	Tyr	Thr	Leu	Ser	Cys	Ala	Phe	Ala	Tyr	Cys	Met	Gly	Gly	Glu	Glu	Arg
AAC	TTC	TAC	TAT	TAC	ACT	CTG	TCC	TGC	GCC	TTC	GCT	TAC	TGT	ATG	GGA	GGA	GAA	GAA	AGA
	850				860				870				880			890			900
															310		320		
Ala	Lys	His	Val	Leu	Phe	Asp	Tyr	Val	Glu	Thr	Cys	Ala	Ala	Pro	Glu	Thr	Arg	Gly	Thr
GCT	AAA	CAC	GTC	CTT	TTC	GAC	TAT	GTT	GAG	ACA	TGC	GCT	GCA	CCG	GAA	ACG	AGA	GGA	ACG
	910				920				930				940			950			960

FIG. 1c

330 340
 Cys Val Leu Ser Gly His Thr Phe Tyr Asp Thr Phe Asp Lys Ala Arg Tyr Gln Phe Gln
 TGT GTT TTA TCA GGA CAT ACT TTC TAT GAC ACA TTC GAC AAA GCC AGA TAT CAA TTC CAG
 970 980 990 1000 1010 1020

350 360
 Gly Pro Cys Lys Glu Leu Leu Met Ala Ala Asp Cys Tyr Trp Asn Thr Trp Asp Val Lys
 GGC CCA TGC AAA GAG CTT CTG ATG GCC GCA GAC TGT TAC TGG AAC ACA TGG GAT GTA AAG
 1030 1040 1050 1060 1070 1080

370 380
 Val Ser His Arg Asp Val Glu Ser Tyr Thr Glu Val Glu Lys Val Thr Ile Arg Lys Gln
 GTT TCA CAT AGA GAT GTT GAG TCA TAC ACT GAG GTA GAG AAA GTA ACA ATC AGG AAA CAG
 1090 1100 1110 1120 1130 1140

390 400
 Ser Thr Val Val Asp Leu Ile Val Asp Gly Lys Gln Val Lys Val Gly Gly Val Asp Val
 TCA ACT GTA GTA GAT TTG ATT GTG GAT GGC AAG CAG GTC AAG GTT GGA GGA GTG GAT GTA
 1150 1160 1170 1180 1190 1200

410 420
 Ser Ile Pro Tyr Ser Ser Glu Asn Thr Ser Ile Tyr Trp Gln Asp Gly Asp Ile Leu Thr
 TCT ATC CCG TAC AGT TCT GAG AAC ACA TCC ATA TAC TGG CAG GAT GGA GAC ATC CTG ACC
 1210 1220 1230 1240 1250 1260

430 440
 Thr Ala Ile Leu Pro Glu Ala Leu Val Val Lys Phe Asn Phe Lys Gln Leu Leu Val Val
 ACG GCC ATC CTA CCT GAA GCT CTT GTC GTT AAG TTC AAC TTT AAG CAG CTC CTT GTA GTT
 1270 1280 1290 1300 1310 1320

450 460
 His Ile Arg Asp Pro Phe Asp Gly Lys Thr Cys Gly Ile Cys Gly Asn Tyr Asn Gln Asp
 CAT ATC AGA GAT CCA TTC GAT GGA AAG ACA TGC GGC ATA TGT GGT AAC TAT AAT CAA GAT
 1330 1340 1350 1360 1370 1380

470 480
 Ser Thr Asp Asp Phe Phe Asp Ala Glu Gly Ala Cys Ala Leu Thr Pro Asn Pro Pro Gly
 TCA ACT GAT GAT TTC TTT GAC GCA GAA GGA GCA TGC GCT CTG ACC CCC AAT CCC CCA GGA
 1390 1400 1410 1420 1430 1440

FIG. 1d

490 500
 Cys Thr Glu Glu Gln Lys Pro Glu Ala Glu Arg Leu Cys Asn Ser Leu Phe Asp Ser Ser
 TGT ACA GAG GAG CAG AAA CCA GAA GCT GAG CGA CTC TGC AAT AGT CTA TTT GAT AGT TCT
 1450 1460 1470 1480 1490 1500

510 520
 Ile Asp Glu Lys Cys Asn Val Cys Tyr Lys Pro Asp Arg Ile Ala Arg Cys Met Tyr Glu
 ATC GAC GAG AAA TGT AAT GTC TGC TAC AAG CCT GAC CGT ATT GCA CCA TGT ATG TAC GAG
 1510 1520 1530 1540 1550 1560

530 540
 Tyr Cys Leu Arg Gly Gln Gln Gly Phe Cys Asp His Ala Trp Glu Phe Lys Lys Glu Cys
 TAT TCC CTG AGG GGA CAG CAA GGA TTC TGT GAC CAT GCT TGG GAG TTC AAA AAA GAA TGC
 1570 1580 1590 1600 1610 1620

550 555
 Tyr Ile Lys His Gly Asp Thr Leu Glu Val Pro Pro Glu Cys Gln ***
 TAC ATA AAG CAT GGA GAC ACT CTA GAA GTA CCA CCT GAA TGC CAA TAAATGAACAAAGATACAG
 1630 1640 1650 1660 1670 1680

AAGCTAAGACTACTACAGCAGAGATAAAAGAGAAGCTGTAGTTCTTCAAAAACAGTATATTTTGATGTACATTTGTT
 1690 1700 1710 1720 1730 1740 1750 1760

1770 1780 1790 1800 1810 1820
 TACTTACATAAAAAATAAATTGTTATTATCATAACGTAAGAGAAAAAAAAAAAAAAAAAAAAA

FIG. 2

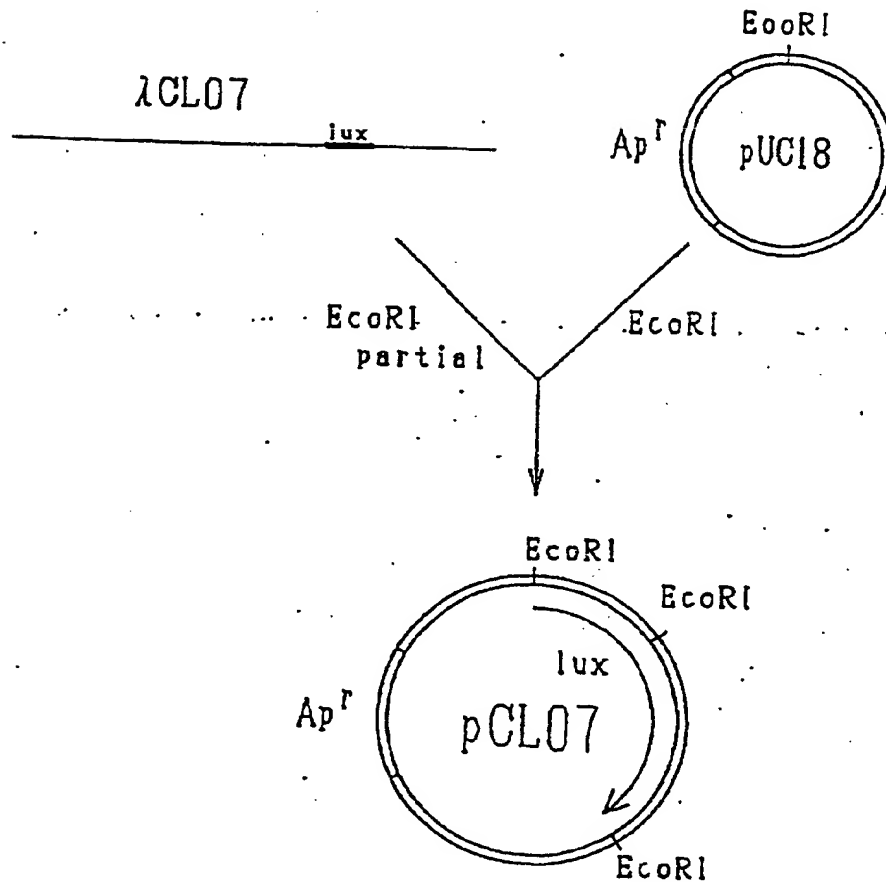


FIG. 3

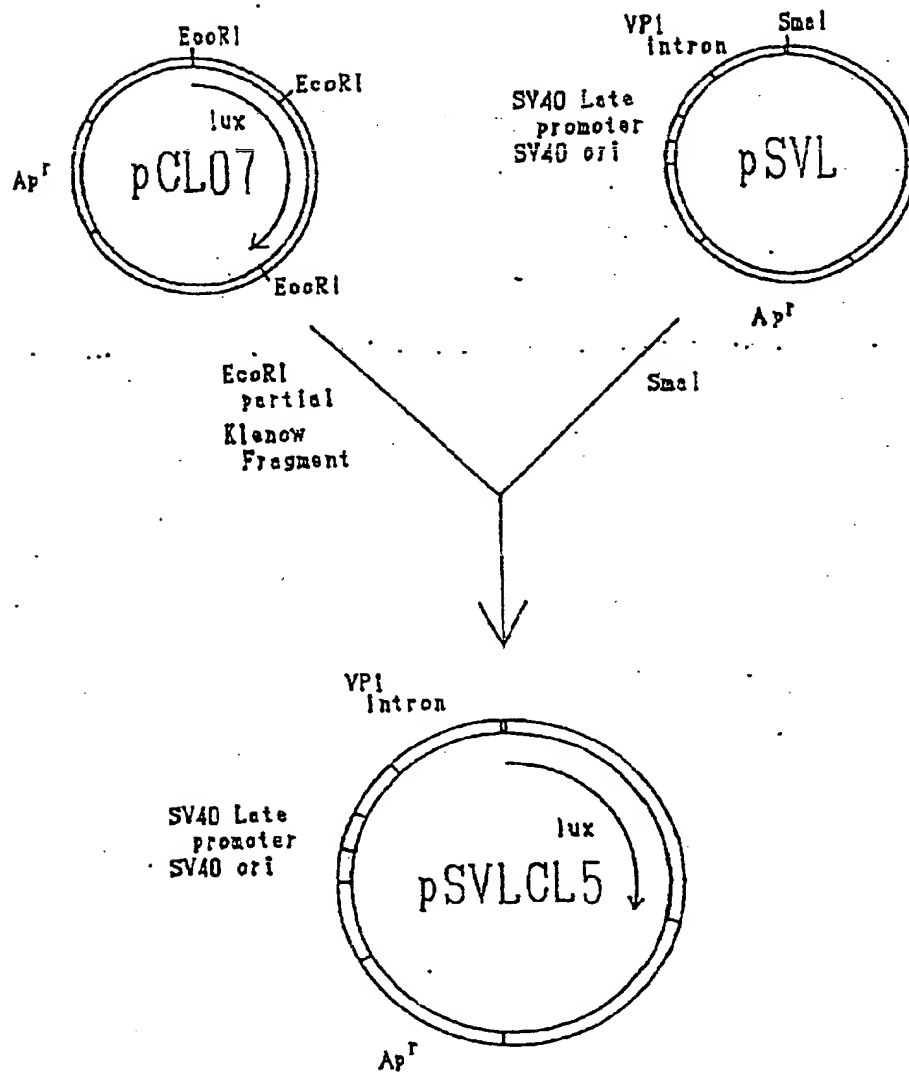


FIG. 4a

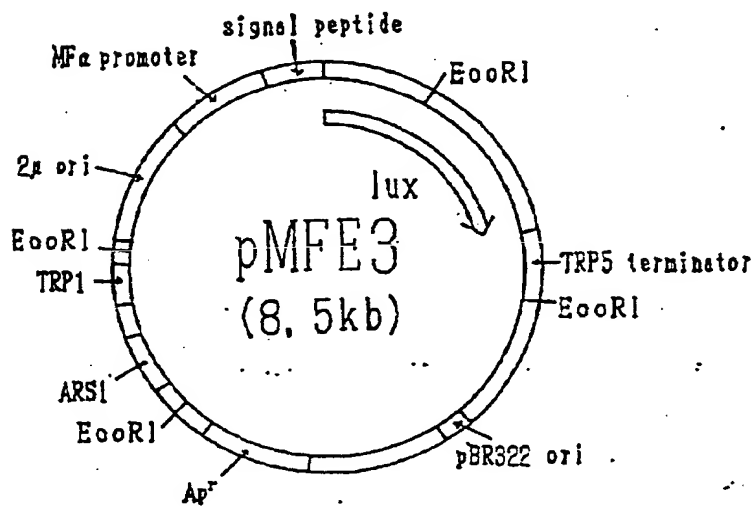


FIG. 4b

		29	30	31	32	33	
(a) pMFE3A	Met....Lys Arg Pro Ser Ser Thr Pro ...						
(b) pMFE3B	Met....Lys Arg --- --- Ser Thr Pro ...						
(c) pMFE3C	Met....Lys Arg --- --- --- Thr Pro ...						
(d) pMFE3D	Met....Lys Arg --- Ser Ser Thr Pro ...						

FIG. 5

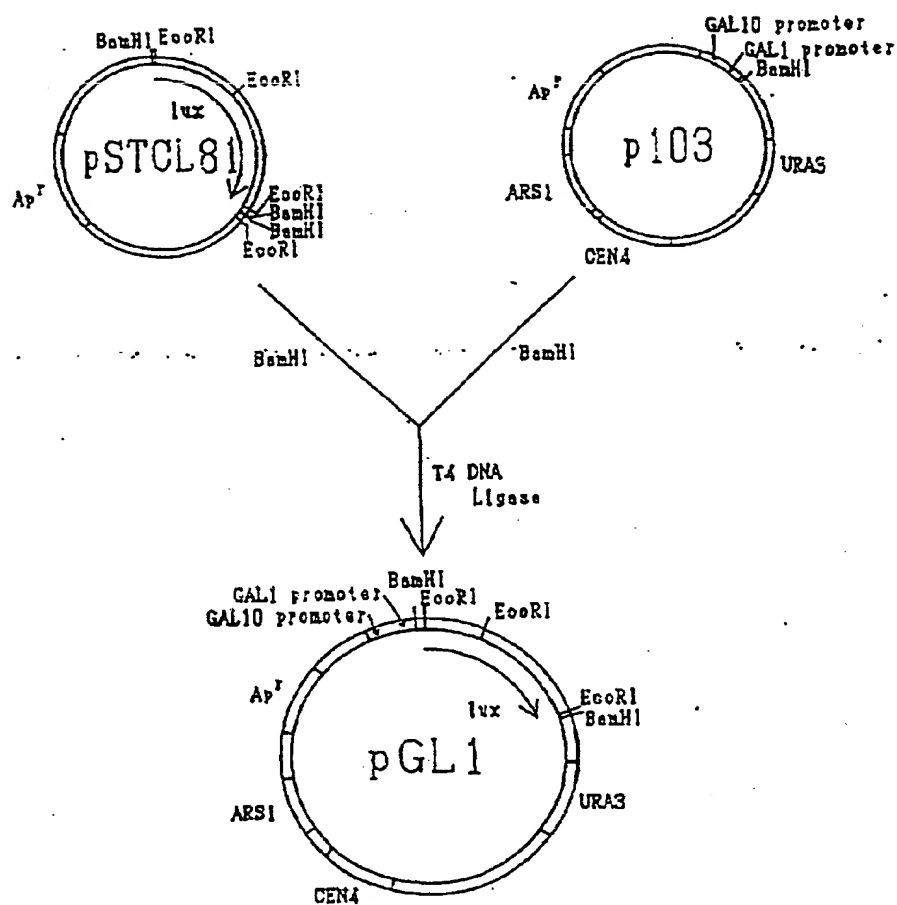


FIG. 6

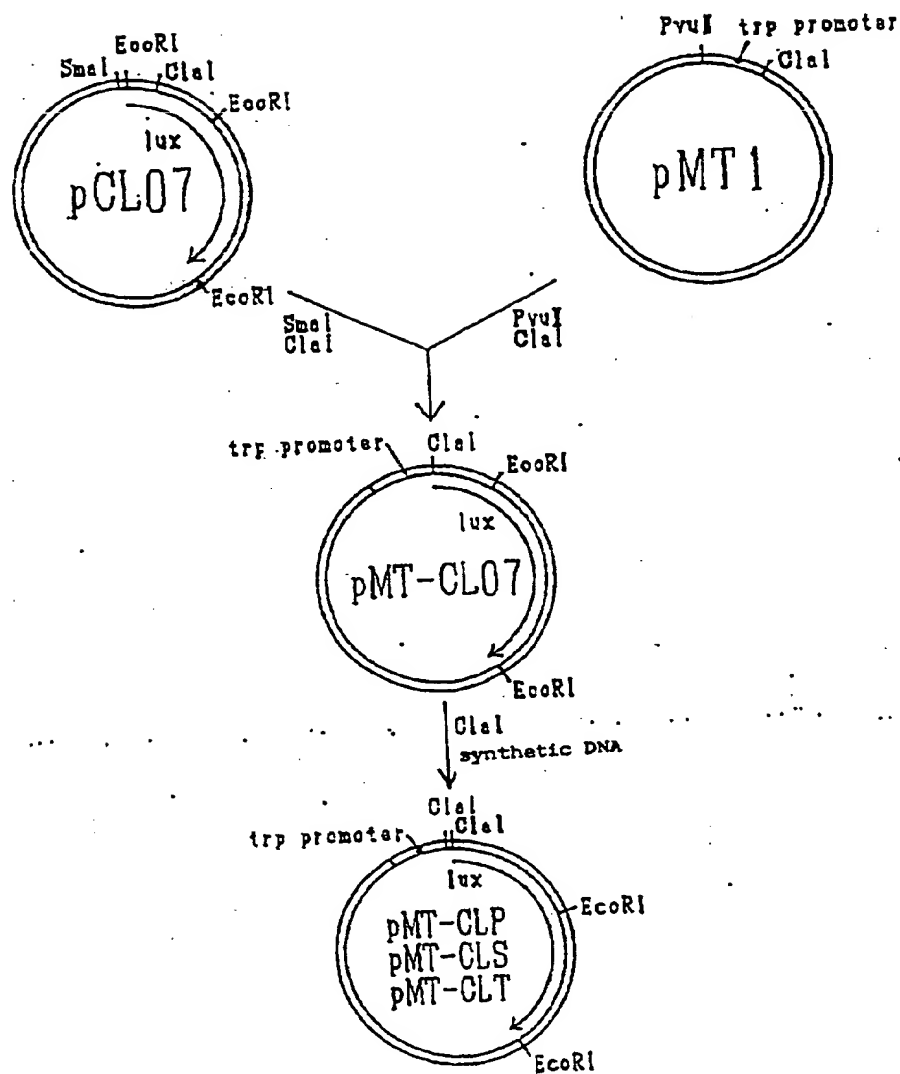


FIG. 1a

Met	Leu	Leu	His	His	Leu	Ser	His	His	Leu	Ala	Tyr	Cys	Val	Thr	Val	Asn	Cys	Gln	Asp
ATG	AAC	CTA	ATA	ATT	CTT	TCT	ATT	ATA	TTT	GCT	TAC	TCT	CTC	ACA	CTC	AAC	TTC	CAA	GAU
15						20					25				30				35
Ala	Cys	Pro	Phe	His	Ala	Glu	Ala	Pro	Ser	Ser	Thr	Pro	Thr	Val	Pro	Thr	Ser	Gln	Glu
GCA	TGT	CTT	CTA	CAA	GGT	CAA	CAA	CGC	TCA	AGT	ACA	CAA	ACA	GGC	CAA	ACA	TCT	TGT	CAA
40						45					50				55				60
Ala	Ser	Phe	Gly	Glu	Cys	Ser	Asp	Thr	Arg	Cys	Ala	Thr	Cys	Lys	Arg	Asp	His	Leu	Ser
GCT	AAA	CAA	CAA	CAA	TGT	ATC	GAT	AGC	AGA	TGT	CAA	ACA	TGT	AAA	CAA	CAAC	ATA	CTA	TCA
65						70					75				80				85
Asp	Gly	Leu	Cys	His	Asn	Ser	Pro	Gly	Lys	Thr	Cys	Cys	Arg	Met	Cys	Gln	Tyr	Val	His
GAC	CGA	CTG	TGT	AAA	AAT	AAA	CCA	GGG	AGC	ATC	TGC	TGT	AGA	ATG	TTC	CAG	TAT	CTA	ATT
90						95					100				105				110
Glu	Cys	Arg	Val	Glu	Ala	Ala	Gly	Tyr	Thr	Arg	Thr	Thr	Tyr	Gly	Lys	Arg	His	Asn	Phe
GAA	TGC	AGA	GGA	GAA	GCT	GCT	CAA	TAT	TTT	AGA	AGC	TTT	TAC	GGC	AAA	AGA	TTT	AAJ	TTT
115						120					125				130				135
Ser	Glu	Pro	Glu	Lys	Tyr	Val	Leu	Ala	Arg	Gly	Thr	Lys	Gly	Gly	Asp	Tyr	Ser	Val	Thr
CAC	CAA	CTT	GGT	AAA	TAT	GTC	GGT	CCA	CCA	ACT	AAA	GCT	GGG	GAA	TGC	TCT	CTA	AGC	
140						145					150				155				160
Leu	Thr	Met	Glu	Asn	Leu	Asp	Gly	Glu	Lys	Gly	Ala	Val	Leu	Thr	Lys	Thr	Thr	Leu	Gln
CTC	ACC	ATC	CTG	AAT	CTA	GAT	CAA	CAC	AAG	CCA	GCT	CTA	CTC	AGC	AAA	ACA	ACA	GTC	CAU
165						170					175				180				185
Val	Val	Gly	Asp	Val	His	Asp	His	Thr	Gln	Ala	Thr	Ala	Asp	Pro	Lys	Tat	Val	Asn	Gly
GTA	GTA	GGA	GAC	GTA	ATA	CAC	ATT	ACT	CAA	GCT	ACT	CCA	GAT	ACT	ACC	ACT	GTC	AAC	CGA
190						195					200				205				210

INTERNATIONAL SEARCH REPORT

International Application No. PCT/JP89/00811

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC		
Int. Cl ⁴ C12N9/02, C12N15/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
IPC	C12N9/02, C12N15/00	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
COMPUTER SEARCH (CHEMICAL ABSTRACTS, BIOSIS DATABASES, EMBL-GDB, LASL-GDB AND NBRF-PDB)		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X, Y	BIOCHEMISTRY, Vol. 13, No. 25, (1974), F.I.Tsuji, et al [Some Properties of Luciferase from the Bioluminescent Crustacean, Cypridina hi/gendorfii] P. 5204 - 5209	1 - 5
A	SCIENCE, Vol. 234, No. 4778, (1986), D.W.Ow, et al [Transient and Stable Expression of the Firefly Luciferase Gene in Plant Cells and Transgenic Plants] P. 856 - 859	6 - 12
A	WO, A1, 88/00617 (BOYCE THOMPSON INSTITUTE FOR PLANT RESEARCH, INC.) 28 January 1988 (28. 01. 88)	6 - 12
* Special categories of cited documents: ** "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "S" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search September 18, 1989 (18. 09. 89)		Date of Mailing of this International Search Report October 2, 1989 (02. 10. 89)
International Searching Authority Japanese Patent Office		Signature of Authorized Officer

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.